GENERON
Advanced Transfer Technologies

SPECIALfinder DETECTION ASSAY HAZELNUT

Cat. N. PAV03A

User Guide

1 - Introduction

Food allergies are an adverse immune response to a food protein that are the most common allergic compound. Food allergies are an important concern for human health; in fact, the presence of specific proteins in any food matrix can cause an allergic reaction IgE mediated. Allergic reactions may have a broad spectrum, which varies on the basis of the individual sensitivity thus generating in some cases severe anaphylactic reactions. Indeed, food correct labeling is of great importance to inform consumers about presence of any allergic substance other than achieve a high level of health protection. Unfortunately, although known allergens can be included in the product (and in the product label) by the food producer, potentially hazardous allergenic residues/contaminants can be present as result of common industrial practices. Cross-contamination between raw materials, production lines or equipment, is a common cause of unwanted allergen transfer between products intended for different scopes.

For all these reasons, developing a detection method for allergic substances ensures customers protection in accordance with food labeling regulations. The SPECIALfinder Hazelnut Detection Assay provide the user with a simple and reliable procedure for the detection of DNA related to species potentially allergenic, in food and feed matrices as well as swabs. Such a detection is an *Indirect Proof* of the potential presence of the Hazelnut proteins into the matrix, being proteins the real allergens.

This assay utilizes the polymerase chain reaction (PCR) to amplify a genetic target typical of the allergenic species. PCR technique can typically detect up to 1-10 copies of the target sequence but the real detection/quantification limit depends on industrial processing degree, sample matrix, DNA extraction and, lastly, on the DNA content of the sample. Genome size of the complex samples under investigation can deeply impact the Limit Of Detection (LOD) also, in addition it does exist a theoretical LOD you cannot go below, given an advised maximum load between 2 and 4 ng DNA/µl reaction mix.

Generon in-house validation: the LOD has been calculated as copy number by means of ddPCR (Droplet Digital PCR), a novel technique capable to count physically the copy number of a selected amplifiable target. With SPECIALfinder Hazelnut an average count of 5-10 copies was obtained. DNA was extracted using Generon ION Force DNA Extractor FAST (Cat. N. EXD001).

The LOD for this assay was experimentally determined between 1 and 0.5 ppm and depends on sample matrix, processing grade, DNA preparation and DNA content.

2 - SPECIALfinder Hazelnut Detection Assay

When used along with GENERase PLUS Mastermix (Cat. N. ENG002) this Real-Time PCR assay detects a specific DNA sequence in the DNA of Hazelnut in less than 1.5 hours. The amplification of the target sequence is measured by the use of a specific fluorescence-labeled probe (FAM).

2.1 - Assay Content

	Box 50 reactions		Box 100 reactions	
	N. vials	Volume (µl)	N. vials	Volume (µl)
SPECIALfinder OLIGO Mix * (OLIGOS and Probe pre-blended mix)	1	250	2	250
Positive Control	1	85	2	85
Negative Control	1	200	1	200

** reagents are supplied with a 5% of extra volume.*

We suggest to use SPECIALfinder Hazelnut Detection Assay along with the following Polymerase Enzyme Ready-to-use mastermix: GENERase PLUS Mastermix (Cat. N. ENG002). When using this GENERase PLUS an additional detection channel (HEX) becomes available to detect the Internal Amplification Control (IAC) to excluding false negative results due to a PCR inhibition.

2.2 - Storage & Expiry information

Expiry date: see date on the packaging, product validity refers to the product kept intact in its original packaging. Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive. Store frozen.

3 – Materials and equipments needed

3.1 – Extraction[1]

Material/Equipment	Source
Extraction Kit	Generon ION Force DNA Extractor FAST (Cat. N. EXD001)
Chemicals: n-esane	Lab Suppliers
Tubes, 50 ml and 15 ml	Generon or other Lab Suppliers
DNAse/RNAse Free Water	Generon or other Lab Suppliers
Vortexer	Generon or other Lab Suppliers
Benchtop Centrifuge for 50 ml Tubes	Generon or other Lab Suppliers
Thermal Water Bath or Block	Generon or other Lab Suppliers
Pipette sets	Generon or other Lab Suppliers
Pipette tips (Barrier)	Generon or other Lab Suppliers
Tube rack for 1.5 ml tubes	Generon or other Lab Suppliers
2.0 and 1.5 ml micro-tubes	Generon or other Lab Suppliers
Micro centrifuge for 1.5-2.0 ml micro-tubes	Generon or other Lab Suppliers
DNA Extraction VACUUM BOX + Vacuum pump or Venturi meter	Generon or other Lab Suppliers

Each step of sample preparation (grinding, transferring, weighing, etc.) must be done according to GLP so that chance of cross-contamination between samples is minimized. It is recommended to use disposable equipment when possible.
If the food samples are not in a powdered or granular form, they should be processed (grinded or blended) before DNA extraction. The majority of DNA extraction methods supports from 20 to 50 mg of starting material. Generon ION Force DNA Extractor FAST (Cat. N. EXD001) allows processing up to 20 grams of starting material in order to maximize sample's lot representation.
Once the sample has been pulverized/homogenized, it can be weighed and the appropriate amount extracted according to DNA extraction method selected. Refer to manufacturer user manual for extraction procedure details.

3.3 – Detection via Real-Time PCR

Material/Equipment	Source
Real-Time PCR System [2]	Generon or other Lab Suppliers
Specialfinder Hazelnut Detection Assay	Generon (Cat. N. PAV03A)
GENERase PLUS Mastermix	Generon (Cat. N. ENG002)
Optical Adhesive Seal and Optical reaction plate or Optical Caps and Strips	Generon or other Lab Suppliers
Micropipette sets	Generon or other Lab Suppliers

(1) Equipment necessary only when ION Force DNA Extractor FAST (Cat. N. EXD001) is used.
(2) The assay can be used with Biorad CFX and MiniOpticon, Stratagene MxSeries, ABI 7300-7500-7900-StepONE-StepONE Plus, Light Cycler 480, Eppendorf realplex, Rotor-Gene Q etc. The assay is not compatible with Roche Light Cycler I and II.

4 – Real-Time PCR detection

4.1 – Reaction setup

I. Allow the reagents to thaw (GENERase PLUS Mastermix, SPECIALfinder OLIGO MIX, Positive Control and Negative Control). Vortex tubes when thawed and spin to collect contents at the bottom of the vial.

II. Mix 250 µl of SPECIALfinder OLIGO Mix with 500 µl of GENERase PLUS Mastermix to prepare SPECIALfinder Working Mastermix (WMX).

III. Vortex briefly and spin down in order to homogenize the mix.

IV. Transfer 15 µl of WMX into each well.

V. Add 5 µl of Negative Control into wells acting as negative control.

VI. Add 5 µl of each sample into wells testing the unknown samples.

VII. Add 5 µl of Positive Control into wells acting as positive control.

VIII. Close wells and ensure no bubbles are present at the bottom of the wells.

IX. Spin briefly optical PCR tubes or plates.

4.2 – Instrument setup

With GENERase PLUS Mastermix set the following parameters on your thermocycler:

I. Total Reaction volume: 20 µl

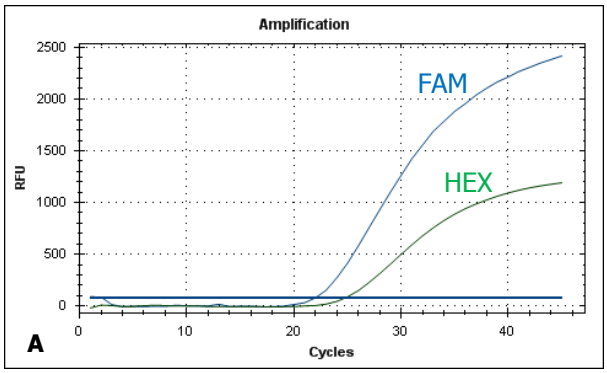
II. Fluorophores/Quenchers: Target Hazelnut (FAM/BHQ1-NFQ); Internal Amplification Control (HEX/BHQ1-NFQ). Depending on your thermocycler, you can replace HEX detector in the plate setting with VIC or JOE in case your own Real Time Platform does not possess the HEX reading channel.

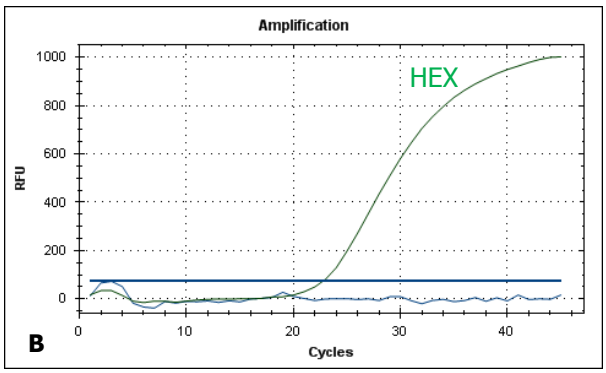
III. Thermal profile:

Step	T (°C)	Duration	Loops
UNG	50	2 min	1
Taq Activation	95	10 min	1
DNA Denaturation	95	15 sec	45
Annealing/Extension + Plate Reading	60	60 sec	

5 – Data Interpretation

Results evaluation must be done according to the analysis software recommended by the Real-Time PCR instrument manufacturer. After performing PCR, each individual sample is analyzed through the instrument software to produce a Cq value (quantification cycle) for each reporter dye. These values are used to determine the presence (Qualitative Test) of allergen into the sample. See below an example of the graphics obtained for a positive (Fig. A) and a negative (Fig. B) control, respectively for the allergen target amplification (blue line) and for the IAC amplification (green line).





After setting the baseline, the analysis outcome should be evaluated following the indications below.

If the following conditions are met:

TEST	Hazelnut (FAM)	Internal Amplification Control (HEX)
Positive Control	+	+
Negative Control	-	+

Then the possible results for any sample are:

TEST	Hazelnut (FAM)	Internal Amplification Control (HEX)
Positive Sample	+	+/-
Negative Sample	-	+
Invalid Sample (inhibited)	-	-

In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph (section 8) for further suggestions.

6 – Inclusivity Panel

Species tested for inclusivity			
Hazelnut (*Corylus avellana*)			

7 – Exclusivity Panel

The following DNA extracts showed no amplification curve in a 20 µl total reaction volume:

Meat			
Beef *(Bos taurus)*	Goat (*Capra hircus*)	Rabbit (*Oryctolagus cuniculus*)	Wild boar (*Sus scrofa*)
Buffalo (*Bubalus bubalis*)	Horse (*Equus caballus*)	Sheep (*Ovis aries*)	
Donkey (*Equus asinus*)	Poultry (*Gallus gallus domesticus*)	Swine (*Sus scrofa domesticus*)	
Duck *(Anas* spp.)	Quail (*Coturnix coturnix*)	Turkey *(Meleagris gallopavo*)	
Crustaceans and Molluscs			
Clam (*Venus gallina*)	Cuttlefish (*Sepia officinalis*)	Mussel (*Mytilus edulis*)	Squid (*Loligo edulis*)
Norway lobster (*Nephrops norvegicus*)	Prawn (*Penaeus vannamei*)		
Hake (*Merluccius merluccius*)	Salmon (*Oncorhynchus kisutch*)	Sea bream (*Sparus aurata*)	Tuna (*Thunnus albacares*)
Vegetables Allergens			
Almond (*Prunus dulcis*)	Durum Wheat (*Triticum durum*)	Oat (*Avena sativa*)	Sesame(*Sesamum indicum*)
Barley (*Hordeum vulgare*)	Kamut (*Triticum turgidum*)	Peanut (*Arachis hypogaea*)	Soft Wheat (*Triticum aestivum*)
Brasilian Walnut (*Bertholletia excelsa*)	Lupine (*Lupinus albus*)	Pecan Nut (*Carya illinoinensis*)	Soybean (*Glycine max*)
Cashew (*Anacardium occidentalis*)	Macadamia Nut (*Macadamia integrifolia*)	Pistachio (*Pistacia vera*)	Spelt (*Triticum monococcum*)
Celery (*Apium graveolens*)	Mustard (*Brassica nigra*)	Rye (*Secale cereale*)	Walnut (*Juglans regia*)
Other			
Ananas (*Ananas comossus*)	Cocoa (*Theobroma cacao*)	Mango (*Mangifera indica*)	Pomelo (*Citrus maxima*)
Apple (*Malus domestica*)	Coconut (*Cocos nucifera*)	Mushroom (*Agaricus campestris*)	Poplar (*Populus* spp.)
Apricot (*Prunus armeniaca*)	Corn (*Zea mays*)	Olive (*Olea europaea*)	Potato (*Solanum tuberosum*)
Banana (*Musa acuminata*)	Cucumber (*Cucumis sativus*)	Onion (*Allium cepa*)	Radish (*Raphanus sativus*)
Basil (*Ocimum basilicum*)	Eggplant (*Solanum melongena*)	Orange (*Citrus aurantium*)	Rapessed (*Brassica napus*)
Broccoli (*Brassica oleracea* var. *italica*)	Fennel (*Foeniculum vulgare*)	Parsley (*Petroselinum crispum*)	Rice (*Oryza sativa*)
Buckwheat (*Fagopyrum esculentum*)	Garlic (*Allium sativum*)	Peach (*Prunus persica*)	Strawberry (*Fragaria x ananassa*)
Carrot (*Daucus carota*)	Grapefruit (*Citrus × paradisi*)	Pear (*Pyrus communis*)	Sunflower (*Helianthus annuus*)
Chard (*Beta vulgaris* var. *cicla*)	Grapes (*Vitis vinifera*)	Peas (*Pisum sativum*)	Tomato (*Solanum lycopersicon*)
Cherry (*Prunus avium*)	Green Beans (*Phaseolus vulgaris*)	Pepper (*Capsicum annuum*)	Zucchini (*Cucurbita pepo*)
Chestnut (*Castanea vulgaris*)	Lemon (*Citrus limonia*)	Pine Nuts (*Pinus pinea*)	
Clementine (*Citrus x clementina*)	Mandarine (*Citrus reticulata*)	Plum (*Prunus domestica*)	

8 – Troubleshooting

I. Concomitant no target or IAC amplification, or amplification plots grossly abnormal. Possible causes and corrective actions:

- An excess of DNA in the target might inhibit the reaction and IAC may be affected due to an excess of DNA and/or PCR inhibitors. Test samples diluted 1:10 and 1:100. Please, use DNase/RNase Free Water to prepare dilutions.
- Inadequate sealing of optical caps/film caused sample evaporation. Redo the analysis using proper tools and proper optical caps/film to secure perfect sealing.
- Did not use the proper consumables. Redo the analysis and use only optical grade 96-well plates and optical adhesive seal or optical 8-well strips and caps.
- Samples were not properly prepared. Remake the sample DNA preps. Ensure that the DNA extraction method is properly performed.

II. Positive Control reactions failed to amplify, but other reactions appear correct (e.g. the IAC is amplified):

- Positive Control DNA was not added to the reaction wells. If other reactions look normal, there may be no need to repeat the run.

III. Negative Control reactions are positive:

- Contamination of the Negative Control vial or the SPECIALfinder PCR mix with SPECIALfinder-positive DNA. Use more care to prevent contamination while handling assay reagents and setting up assays.

In case support is needed contact Generon at: support@generon.it

9 – Disclaimers

The product is intended for research use only. Generon makes no warranty of any kind, either expressed or implied, except that the materials from which its products are made of standard quality. If any materials are defective, Generon will provide a replacement product. Generon shall not be liable for any damages, including special or consequential damage, or expense arising directly or indirectly from the use of this product. Please do not interchange components between assays of different lot numbers. This assay is designed to be used by laboratory personnel following the common molecular biology precautions.

Quick Reference Guide

Product Line:	SPECIALfinder
Part Number:	PAV03A
Type:	Qualitative
Storage:	Frozen
Execution time:	about 120 minutes
Expiry date:	see date on the packaging, product validity refers to the product kept intact in its original packaging and constantly under suitable temperature conditions as mentioned above.

Assay Box Content

	Box 50 reactions		Box 100 reactions	
	N. vials	Volume (µl)	N. vials	Volume (µl)
SPECIALfinder OLIGO Mix (OLIGOS and Probe pre-blended mix)	1	250	2	250
Positive Control	1	85	2	85
Negative Control	1	200	1	200

All reagents are supplied with a 5% of extra volume.

Not Provided Article: GENERase PLUS Mastermix (cat. N. ENG002) or equivalent.

Reaction Set-Up

Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive.

Before setting the analysis, we strongly advise to leave the reagents to warm up at room temperature. Vortex briefly OLIGO mix, afterwards spin to collect contents at the bottom of the vials. Spin GENERase PLUS Mastermix before opening it.

Prepare SPECIALfinder WORKING Mastermix by adding 250 µl of SPECIALfinder OLIGO Mix into each tube prefilled with 500 µl of GENERase PLUS Mastermix (Cat. N. ENG002) in order to obtain a single volume of 750 µl of SPECIALfinder WORKING Mastermix. Vortex briefly SPECIALfinder WORKING Mastermix with the aim of homogenizing the mix and excluding $MgCl_2$ gradient that could impair the results. Spin to collect contents at the bottom of the vial *(Note: label GENERase PLUS vials with target name after OLIGO Mix addition)*. Vortex briefly Positive Control and samples before proceeding further, spin to collect contents at the bottom of the vial.

Transfer SPECIALfinder WORKING Mastermix and samples into the plate as follows:

Reagents per well	Volume
Unknown Sample	5 µl
Positive Control Negative Control	
SPECIALfinder WORKING Mastermix	15 µl
Final Volume	20 µl

Detector Setup

Target	Reporter Dye	Quencher Dye
Hazelnut	FAM	BHQ1-NFQ
IAC (Internal Amplification Control)	HEX (*)	BHQ1-NFQ

(*)According to your thermocycler you can replace HEX detector in the plate setting with VIC or JOE in case your own Real Time Platform does not possess the HEX reading channel.

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Thermal cycling

Step	T (°C)	Duration	Loops
UNG	50	2 min	1
Taq Activation	95	10 min	1
DNA Denaturation	95	15 sec	45
Annealing/Extension + Plate Reading	60	60 sec	

The thermal profile presented above was optimized for GENERase PLUS Mastermix (Cat. N. ENG002).

Results analysis

If the following conditions are met:

TEST	Hazelnut (FAM)	Internal Amplification Control (HEX)
Positive Control	+	+
Negative Control	-	+

Then the possible results for any sample are:

TEST	Hazelnut (FAM)	Internal Amplification Control (HEX)
Positive Sample	+	+/-
Negative Sample	-	+
Invalid Sample (Inhibited)	-	-

In case of inhibition DNA isolation and purification for the sample need to be improved or you may need to dilute your sample before performing a new test. Refer to the Troubleshooting paragraph, section 8 in the User Guide, for further suggestions.

Warning and Precaution

Please, do not interchange components of assays with different lot numbers. This assay is designed to be used by laboratory personnel following the common molecular biology precautions (GLP).

Disclaimer

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The product was internally tested by our quality control. Any responsibility is waivered if the warranty of quality control does not refer to the specific product. The user is personally responsible for data that he will obtained and/or he will supply to third parties using this assay. Once the sealed package is open the user accepts all the conditions without fail; if the package is still sealed the product can be returned and the user can be refunded.